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Transmissibility of Naturally Developed Sulfonamide Resistance in Shigella

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SUMMARY

Genetic analyses were made of the non-transmissible Sa-resistance factor of *Shigella flexneri* 2a-30(Sa) which remained after other antibiotic-resistance factors had been eliminated from a multiple drug resistant *Shigella* strain 2a-30(Sm^{1000}, Cm, Tc, Sa) by acridine treatment.

The Sa-factor was reactivated in transmissibility by introduction of the R(Sm^{125})-factor from *Shigella flexneri* 2a-50(Sm^{125}, Sa). Thus Sa-resistance could be transferred to *Shigella flexneri* 3a-18gal$^-$ together with the R(Sm^{125})-factor. In 3a-18gal$^-$(Sm^{125}, Sa) there was loss of Sm-resistance occuring spontaneously or by acridine treatment. In the 3a-18gal$^-$(Sa) thus obtained, Sa-resistance was transmissible and it was resistant to acridine treatment. This is the first demonstration of an episomic R-factor capable of transferring only Sa-resistance. The remarkable characteristics of the R(Sm^{125})-factor are described.

INTRODUCTION

The multiple drug resistance of *Escherichia coli* and dysentery bacilli is transmissible to other sensitive enteric bacteria as found independently by Ochiai *et al.* (1959) and by Akiba *et al.* (1960). They and several other workers (Nakaya *et al.*, 1960; Mitsuhashi *et al.*, 1960; Watanabe *et al.*, 1961a) studied the mechanism of transmission of drug resistance. Transfer was found to be independent of endogenotic chromosomal markers and mediated by a new episomic factor. It has very recently been agreed to call it the R-factor by workers in this country.

The R-factor could be eliminated by growing cells in a medium containing an acridine dye. On elimination of the R-factor from some strains resistant to Sm, Cm, Tc and Sa as reported by Watanabe *et al.* (1961b) and by Harada *et al.* (1961), strains were found in which resistance to Sm, Cm and Tc could be elimi-

Abbreviations: Symbols in parentheses are as follows.
Sm: resistance to streptomycin
Sm^{125}, Sm^{1000}: resistance up to 125 μg/ml of streptomycin and 1000 μg/ml of streptomycin, respectively
Cm: resistance to chloramphenicol
Sa: resistance to sulfonamide (sulfathiazole)

nated but not resistance to Sa. A strain such as this, which was only resistant to Sa, was no longer capable of transferring Sa-resistance to a Sa-sensitive recipient. However, when the R-factor (Sm^{1000}, Cm, Tc, Sa) was transmitted to a sensitive strain and then this strain was treated with an acridine dye, Sa-resistance was also eliminated together with resistance to three other antibiotics. The present authors also found Sm-, Cm-, Tc- and Sa-resistant strains of the same character during routine examination of dysentery bacilli. Attempts were made to reactivate the transmissibility of the Sa-factor. After the introduction of an R-factor carrying only Sm-resistance from *Shigella flexneri* type 2a strain 2a-50(Sm^{125}, Sa), the Sa-factor recovered transmission potency. The present paper describes the details of these experiments and further analyses of the nature of the Sa- and Sm^{125}-factor.

MATERIALS AND METHODS

1. *Bacterial strains*

In this study freshly isolated wild strains of dysentery bacilli and their derivatives were used.

1) *Wild type strains*: 2a-30(Sm^{1000}, Cm, Tc, Sa) and 2a-50 (Sm^{125}, Sa) were naturally developed drug-resistant strains of type 2a of *Shigella flexneri* and 3a-18 was a drug-sensitive strain of type 3a of *Shigella flexneri*. They were separately isolated from different sources and have been stocked in the Osaka Municipal Hygiene Laboratory. Their characteristics are summarized in Table 1.

2) *Derivatives of the wild strains:*

a. 2a-30(Sa) was obtained from 2a-30(Sm^{1000}, Cm, Tc, Sa) by growing the cells overnight in the presence of 10 μg/ml of trypaflavine (Hirota, 1957; Watanabe, 1961b). This strain kept the same level of Sa-resistance as the original strain.

b. 3a-18gal$^-$, non-fermenting mutant for galactose, was isolated from an ultraviolet-treated suspension of a 3a-18 culture. Thereafter, the original strain was designated as 3a-18gal$^+$. The mutated gene locus has not been studied yet, but gal$^-$mutation was associated with a change in sensitivity to some bacteriophages and colicines. 3a-18gal$^+$ was insensitive to T_1, T_4 and P_1 phages and colicines K and X, whereas 3a-18gal$^-$ was very sensitive to the above phages and colicines. Other characters of the two strains are shown in Table 1.

Table 1. List of Strains Used

Strain	Serotype	Fermentation markers	Resistance to drugs (μg/ml) Sm	Cm	Tc	Sa
2a-30 (Sm^{1000}, Cm, Tc, Sa)	flex. 2a	Mtl$^+$Sorb$^-$Mal$^+$Gal$^+$	1000	100	100	1000
Acridine treated 2a-30 (Sa)	flex. 2a	Mtl$^+$Sorb$^-$Mal$^+$Gal$^+$	1.0	0.5	6.0	1000
2a-50 (Sm^{125}, Sa)	flex. 2a	Mtl$^+$Sorb$^-$Mal$^+$Gal$^+$	125	1.0	6.0	1000
3a-18gal+	flex. 3a	Mtl$^-$Sorb$^+$Mal$^-$Gal$^+$	1.5	0.5	6.0	6.0
3a-18gal−	flex. 3a	Mtl$^-$Sorb$^+$Mal$^-$Gal$^-$	1.5	0.5	6.0	6.0
Infected 3a-18gal+(Sm^{125})	flex. 3a	Mtl$^-$Serb$^+$Mal$^+$Gal$^+$	125	0.5	6.0	6.0

The symbols represent: Mtl=mannitol, Sorb=sorbitol, Mal=maltose, Gal=galactose, Sm=streptomycin, Cm=chloramphenicol, Tc=tetracycline, Sa=sulfathiazole

c. 2a-30(Sa, Sm^{125}) was derived from 2a-30(Sa) by the following procedures. A culture of 3a-18gal$^-$ was mixed with 2a-50(Sm^{125}, Sa) and Sm and Sorb markers selected. The Sm-resistant conjugant 3a-18gal$^-$(Sm^{125}) obtained was again mixed with 2a-30(Sa) and Sa and Sm markers selected. The conjugant obtained was termed 2a-30(Sa, Sm^{125}). Its resistance to Sm was of the same level of 125 μg as that of 2a-50(Sm^{125}, Sa).

2. *Conjugation experiments*

Resistant donor cells and sensitive recipient cells were mixed in nutrient broth at equal initial concentrations of *ca.* 3×10^8 cells per ml. The mixture was incubated at 37° with gentle shaking. At various intervals samples were withdrawn, suitably diluted and spread on selective media for conjugants. When Sm-resistant colonies were to be selected, nutrient agar containing Sm at a final concentration of 25 μg per ml was used as the basal medium. When conjugants were to be selected for Sa, casamino acid medium, consisting of $Na_2HPO_4 \cdot 12H_2O$ 2.5g, KH_2PO_4 0.35g, NaCl 5.0g, $MgSO_4 \cdot 7H_2O$ 0.1g, casamino acid 2.0g, l-tryptophan 0.01g, nicotinic acid 0.01g, thiamine 0.01g, agar 15g and H_2O 1000 ml, was used as the basal medium and sulfathiazole was added at a final concentration of 100 μg per ml. Before spreading on casamino acid medium, the mixed culture was washed free of nutrients. Sugar was added to all the media at a final concentration of 1.0 per cent, with bromthymolblue at a final concentration of 0.0012 per cent, so as to distinguish the parental strains from each other.

On inoculated selective media, recipient cells, which had received resistance factor (R-factor), were recognized after one or two days' incubation at 37°C as sugar fermenting colonies on a non-fermenting background of the donor strain.

To follow the kinetics of R-factor transfer, the interrupted conjugation method described by Hayes(1957) was applied. Parental cells were mixed and incubated in the usual way. At intervals, 1.0 ml samples of the mixture were rapidly transferred to 1.0 ml of T_1 phage suspension in nutrient broth (about 10^{10} particles per ml) preincubated at 37°C. The donor was sensitive but the recipient was resistant to this. The phage treated samples were always spread on selective media.

3. *Assay of drug resistance*

The assay of resistance to antibiotics was performkd on nutrient agar containing graded concentrations of each antibiotic. The inoculation was made by streaking a loopful of saline suspension of the culture to be tested onto the agar. When testing sulfonamide resistance, casamino acid agar supplemented with 1 per cent glucose was used and sulfathiazole was added in graded concentrations. The inoculated agar plates were incubated at 37°C for 24 hours before scoring, whereas casamino acid agar plates were incubated for 48 hours.

RESULTS

1. *Reactivation of transmissibility of Sa-resistance*

As the inefficiency of 2a-30(Sa) in transferring the Sa-factor was due to loss of other antibiotic resistance factors, it seemed possible that transmitting capacity of this strain would be reactivated by transfer of another antibiotic resistance factor. For this purpose several R-factors not carrying Sa-resistance were directly or indirectly transferred to 2a-30(Sa) but in only one case was the object achieved. In this case the R(Sm^{125})-factor of 3a-18gal$^+$(Sm^{125}), which had originally been transmitted from 2a-50(Sm^{125}, Sa), was transferred to 2a-30(Sa), and the 2a-30(Sm^{125}, Sa) thus obtained was tested for its ability to transmit Sa- and Sm-resistance by growing it with 3a-18gal$^+$ and selecting Sa and sorbitol markers. As seen in Table 2, Sa-resistance became transferable together with the R(Sm^{125})-

factor when conjugant colonies were selected with Sa. Thus it was shown that the introduction of the R(Sm^{125})-factor originating from 2a-50(Sm^{125}, Sa) made the nontransferable Sa-resistance transferable.

Table 2. Transfer of R-factor from 2a-30 (Sm^{125}, Sa) to 3a-18gal+ in Conjugation Interrupted by T_1 phage

Time of incubation of mixed culture before phage addition	No. of colonies per 0.1ml sample when selected for			Viable counts of recipient per 1 ml sample on nutrient agar
	Sm 25 μg/ml	Sa 100 μg/ml	Sm 25μg/ml Sa 100μg/ml	
1 min.	2100	1100	0	3.0×10^8
30 min.	2100	1100	0	4.5×10^8
1 hr.	1700	1200	0	6.2×10^8
2 hr.	800	1300	0	8.9×10^8
3 hr.	540	1300	0	1.7×10^9

2. *Two genotypes of naturally developed streptomycin resistance*

As described above, the degree of streptomycin resistance of 2a-30(Sm^{1000}, Cm, Tc, Sa) differed from that of 2a-30(Sm^{125}, Sa). Both resistance factors can be transferred by conjugation, without any change in their resistance levels. Furthermore, an additional difference was found in the kinetics of transfer of resistance facfors. Three ml of each donor culture were incubated with 3 ml of recipient culture. Samples were taken at intervals (1 min, 1, 2 and 3 hours) from each mixture and spread on nutrient agar containing 25 μg of streptomycin and 1 per cent sorbitol. The sorbitol positive colonies were counted. The results are shown in Table 3.

Table 3. Comparison of the Appearance of Conjugant Colonies on Streptomycin Agar in Crossing of 2a-30 (Sm^{125},Sa) × 3a-18gal + and 2a-30 (Sm^{1000}, Cm, Tc, Sa)×3a-18gal+

Time of incubation of mixed culture before plating	No. of colonies on Sm-agar on incubation of 0.1 ml samples, using the following strains as donors	
	2a-30 (Sm^{125}, Sa)	2a-30 (Sm^{1000}, Cm, Tc, Sa)
1 min.	2200	0
1 hr.	2000	0
2 hr.	1100	24
3 hr.	480	1200

In conjugation experiments between 2a-30(Sm^{125}, Sa) and 3a-18gal$^+$, cultures mixed just before plating gave a considerable number of conjugant colonies on selective media containing streptomycin. When 2a-30(Sm^{1000}, Cm, Tc, Sa) was tested with the same recipient, a culture mixed just before plating yielded no conjugant colonies and there was always 120 minutes lag before the appearance of the conjugant. The Sm-resistance factor from 2a-30(Sm^{125}, Sa) could be very rapidly

expressed after transfer, whereas that from 2a-30(Sm^{1000}, Cm, Tc, Sa) was not fully expressed until 120 minutes later. This would indicate the presence of the two kinds of genotypes concerned with streptomycin resistance in *Shigella*. These two R-factors must be different and distinct entities.

3. *Time analysis of conjugation between 2a-30(Sm^{125}, Sa) and 3a-18gal$^+$*

As the Sm-resistance of 2a-30(Sm^{125}, Sa) could be transferred and expressed very rapidly, the transfer of Sa-factor of 2a-30(Sm^{125}, Sa) was studied as a function of time by interrupting conjugation with T_1 phage using 3a-18gal$^+$ as a recipient. The results are also shown in Table 2.

Samples treated with phage immediately after mixing the two cultures gave a considerable number of conjugants on agar containing streptomycin as well as on agar containing sulfonamide. So it is evident that the introduction of Sa- and Sm-factors into the sensitive cells has taken place simultaneously and instantaneously, and the two resistance mechanisms are phenotypically expressed at the same time.

The conjugants obtained on the two agar plates were tested for their response to other drugs. In these tests, colonies on agar plates containing one of the drugs were suspended in saline and streaked on the same type of agar plates. The colonies were again suspended in saline and streaked on the two kinds of agar plate containing the two drugs in graded concentrations. In the experiments 200 conjugant colonies were picked from each plate inoculated with the samples removed at each time and tested for their response to other drugs. The conjugants which grew on sulfonamide agar were all resistant to both streptomycin and sulfonamide. On the other hand, all the conjugants which grew on streptomycin agar showed resistance to streptomycin only. Therefore the resistance pattern of the conjugants differed depending on the kind of drug used for selection and doubly resistant conjugants could be obtained on sulfonamide agar but not on streptomycin agar. This was true for samples taken at each of the time intervals after phage treatment. Casamino-sorbitol-agar plates containing streptomycin together with sulfonamide never yielded conjugant colony, in accordance with the fact that no doubly resistant conjugants arose in the presence of streptomycin. Once selected with Sa, however, doubly resistant conjugants retained the Sa-factor and could be propagated on Sm-Sa-agar. So it seems likely that streptomycin hindered the development of the phenotypic expression of the introduced Sa-factor.

Another interesting finding was with regard to the yields of Sm-conjugants, as shown in the table. A maximal yield of the Sm-conjugant was achieved from samples treated immediately after mixing. The yields remained constant for 30 minutes. After 30 minutes the yield decreased progressively with increase in the incubation period in spite of the growth of the recipient cells in the mixed culture. This finding is discussed below.

4. *Spontaneous loss of Sm-resistance in conjugant 3a-18gal$^+$(Sm^{125}, Sa)*

Spontaneous loss of Sm-resistance was noticed occasionally in one of the stock cultures of 3a-18gal$^+$(Sm^{125}, Sa), which had been previously shown to be resistant to both streptomycin and sulfonamide. This phenomenon was studied.

A culture of 3a-18gal$^-$(Sm^{125}, Sa), which had been stocked after purification by three successive single colony isolations on Sm-Sa-casamino acid agar plates, was spread on fresh Sm-Sa-casamino acid agar plates. One of the colonies that arose after two days incubation was suspended in saline and 0.1 ml aliquots of a 1 : 10^5 dilution were spread on three plates of each medium (nutrient agar, nutrient agar containing 25 μg streptomycin per ml, casamino acid agar containing 100 μg sulfathiazole per ml) to compare the survival on each medium. The average counts on the three plates at different times are shown in the second column of Table 4. Colonies from each medium were picked at random and suspended in saline. A loopful of each suspension was streaked on nutrient agar containing 25 μg of streptomycin and also on casamino acid agar medium containing 100 μg of sulfathiazole, to classify the resistance patterns. The percentages of colonies with different resistance patterns are also shown in Table 4.

Table 4. Stability of R-factor in Cultures of 3a-18gal$^-$ (Sm^{125}, Sa)

medium	Survival counts	Percentage of colonies having resistance patterns				Total colonies tested
		(Sm, Sa)	(Sm)	(Sa)	sensitive	
Nutrient agar	339	88	0	12	0	600
Streptomycin agar	287 (84.7%)	100	0	0	0	600
Sulfathiazole agar	330	89	0	11	0	600

As seen in Table 4 survival counts on Sa-casamino acid agar were almost equal to those on the nutrient agar, but survivals on Sm-agar were appreciably less and about 15 per cent less than on nutrient agar. As there was no evidence of a difference in colony size or delayed colonial growth on Sm-agar, the reduction in survival on Sm-agar could be considered to reflect the heterogeneity in Sm-resistance of the population. To prove this, colonies from nutrient agar plates were tested for their response to streptomycin and sulfonamide. About 12 per ceut of these colonies were found to be sensitive to streptomycin. The per cent was roughly equal to the reduction rate. The frequency of loss of Sm-resistance was found to be about 15 per cent. Of interest in the present case is the fact that there was no evidence of loss of Sa-resistance.

The instability of Sm-resistance was further studied on conjugant 3a-18gal$^+$-(Sm^{125}) which had been obtained from 3a-18gal$^+$ by conjugation with 2a-50-(Sm^{125}, Sa) and selection with Sm. A loss of Sm-resistance was constantly observed, although with a low frequency of 5~10 per cent. Therefore Sm-resistance was unstable whether it was transmitted to the recipient 3a-18gal$^+$ together with or separately from the Sa marker. This would account for the reduction in yield of Sm-conjugants on prolonged incubation of mixed cultures of the parents, as shown in Table 2.

5. *Loss of the resistance in relation to the intrinsic character of the recipient*

To elucidate the cause of the loss of Sm resistance in 3a-18gal$^+$(Sm^{125}, Sa), studies were made of whether the instability of Sm-resistance was inherent to the marker itself or related in some way to the intrinsic character of the recipient. 3a-18gal$^-$, a substrain of 3a-13gal$^+$, was chosen as the recipient for this comparative study.

2a-30(Sm^{125}, Sa) was grown with 3a-18gal$^-$, and conjugants were selected with streptomycin. A comparison was made of these with the conjugants of the same donor and 3a-18gal$^+$. Samples were taken at intervals from each mixed culture and spread on nutrient agar containig 25 μg of streptomycin. The surface of the agar was spread with T_1 phage. The results are shown in Fig. 1.

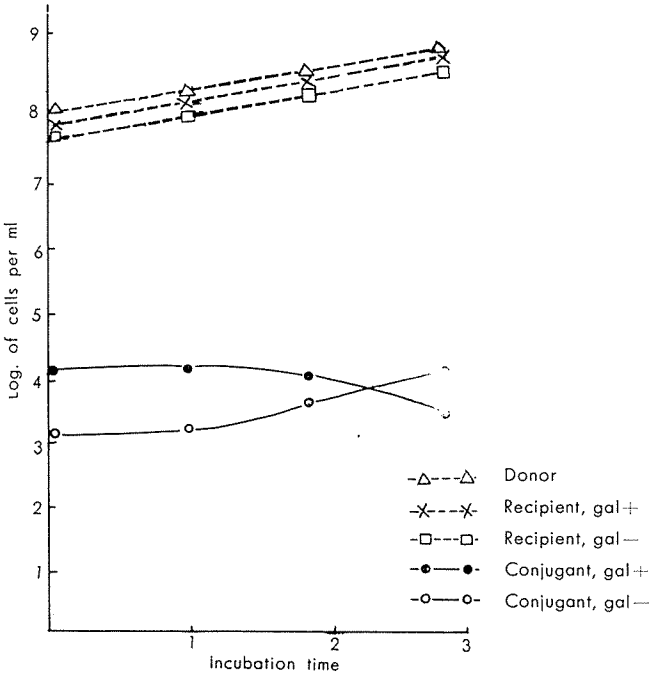

Fig. 1. Sm-conjugants between 2a-30(Sm^{125}, Sa)×3a-18gal+ and 2a-30(Sm^{125}, Sa)×3a-18gal—

Unlike the results obtained with 3a-18gal$^+$, the Sm-conjugants of 3a-18gal$^-$ increased in number with increasing time of incubation. This can be explained from results obtained in following experiments on the stability of the transferred Sm-resistance of 3a-18gal$^-$(Sm^{125}, Sa). The experimental procedure was the same as in the case of 3a-18gal$^+$(Sm^{125}, Sa). In contrast to 3a-18gal$^+$(Sm^{125}, Sa), in 3a-18gal$^-$(Sm^{125}, Sa) the Sa-resistance only was found to be unstable and the Sm-

resistance was quite stable, as shown in Table 5. So it is conceivable that certain host factors play an important role in the mechanisms underlying the loss of the resistance factors.

Table 5. Stability of R-factor in Culture of 3a-18gal$^-$ (Sm^{125}, Sa)

medium	Survival counts	Percentage of colonies having resistance patterns				Total colonies tested
		(Sm, Sa)	(Sm)	(Sa)	sensitive	
Nutrient agar	240	98	2	0	0	600
Sulfathazole agar	216 (90.1%)	100	0	0	0	600
Streptomycin agar	237	97	3	0	0	600

6. *Stability of acridine treated Sa-resistance in strain 2a-30(Sa) and the influence of introduced Sm-resistance*

In previous experiments it was shown that the stability of Sa-or Sm-resistance depended on the intrinsic character of the recipient cells, *i.e.* gal$^+$ or gal$^-$. Further studies were made on the stability of Sa-resistance in 2a-30(Sa) obtained from 2a-30(Sm^{1000}, Cm, Tc, Sa) by acridine treatment and in 2a-30(Sm^{125}, Sa) obtained from 2a-30(Sa) by conjugation with 3a-18gal$^+$(Sm^{125}).

One of the colonies of 2a-30(Sa) on Sa-casamino acid agar was suspended in saline and aliquots of varying dilutions were spread on both nutrient agar and Sa-casamino acid agar. There was no appreciable difference in their survival counts on the two media. To check this, numerous colonies on nutrient agar were tested for their response to Sa. No colonies were found with a major population sensitive to Sa, as shown in Table 6.

Table 6. Stability of Sa-factor of 2a-30 (Sa) and 2a-30 (Sm^{125}, Sa)

Culture	medium	Survival counts	Percentage of colonies having resistance patterns				Total colonies tested
			(Sm, Sa)	(Sm)	(Sa)	sensirtve	
2a-30 (Sa)	Nutrient agar	260	0	0	100	0	500
	Sulfathiazole agar	258					
2a-30 (Sm, Sa)	Nutrient agar	236	95	3	1	1	500
	Sulfathiazole agar	227					

Tests were carried out on 2a-30(Sm^{125}, Sa) in the same way as in the case of 3a-18gal$^+$(Sm^{125}, Sa). As seen in Table 6, the Sa-resistance was lost either together with or independently of the Sm-resistance. These findings indicate that

the R(Sm^{125}) factor could render the otherwise stable Sa-resistance labile.

7. *Transmissibility of Sa-resistance released from combination with Sm-resistance*

As otherwise non-transmissible Sa-resistance of 2a-30(Sa), retained after acridine treatment, could be transmitted in the presence of R(Sm^{125})-factor, attempts were made to see whether the Sa-resistance of 3a-18gal$^+$(Sm^{125}, Sa) thus rendered transmissible still retained its ability after having been spontaneously released from the R(Sm^{125})-factor. Thus 3a-18gal$^+$(Sa) was compared with 2a-30(Sa), released from the R(Sm^{125})-factor.

2a-30(Sa) and 3a-18gal$^+$(Sa) were each grown with 3a-18gal$^-$ and the mixtures were spread on casamino acid-galactose-Sa-agar to select the Sa-resistant conjugants of 3a-18gal$^-$. Conjugant colonies could be obtained from the mixture containing 3a-18gal$^+$(Sa), but not from the mixture containing 2a-30(Sa). As the Sa-resistance of 2a-30(Sa) was non-transmissible and resistant to acridine dye and the Sa resistance of 3a-18gal$^+$(Sa) became transmissible, the fate of the latter was studied after treatment with an acridine dye. In spite of extensive efforts, elimination of Sa resistance from 3a-18gal$^+$(Sa) was not accomplished. Each of the five colonies on nutrient agar arising from overnight culture of 3a-18gal$^+$(Sa) in broth containing trypaflavine at a final concentration of 10 μg per ml, was purified. After confirmation for their response to Sa, each culture was grown with 3a-18gal$^-$ to look for Sa-resistant gal$^-$ colonies. It was found that all the colonies transferred Sa-resistance.

3a-18gal$^+$(Sa) could also be obtained from 3a-18gal$^+$(Sm^{125}, Sa) by acridine treatment. In this case about 20 per cent of the colonies obtained had lost Sm-resistance after treatment, and half of them could be regarded as having arisen spontaneously. The other half must be regarded as having been due to acridine. The eighteen colonies thus obtained, which were resistant to Sa only after acridine treatment, were tested for their capacity to transmit Sa and all were found to be potent. It is conceivable that colonies arising spontaneously as well as those due to acridine were among these 18 colonies. Therefore, it can be assumed that the loss of Sm-resistance from 3a-18gal$^+$(Sm^{125}, Sa) renders the Sa-resistance factor transferable, irrespective of the cause of the loss of Sm-resistance.

DISCUSSION

There are three possible explanations of the genetic structure of strain 2a-30(Sm^{1000}, Cm, Tc, Sa). First, the strain may contain two kinds of genetic factors of Sa-resistance capable of expressing the same degree of resistance to Sa, one of which is exogenotic and carried by the R-factor together with the genetic factors of Sm-, Cm- and Tc-resistance. The other is endogenotic, in the chromosome. Therefore, the Sa-resistance still retained after acridine treatment must be due to the endogenotic factor. Second, the strain may have no endogenotic Sa-resistance

gene and contain two exogenotic R-factors, the one of which carries Sm-, Cm- and Tc-resistance and the other Sa-resistance, and the two R-factors are coupled together. After acridine treatment of 2a-30(Sm^{1000}, Cm, Tc, Sa), the coupling is broken and the second R-factor carrying Sa-resistance and defective in the transmissibility is left in the 2a-30(Sa). The third possibility is a slight modification of the second. The strain may contain two exogenotic R-factors, one carring Sm-, Cm-, Tc- and Sa-resistance and the other carrying Sa-resistance only and the latter is defective in transmissibility. In addition, the degree of Sa-resistance is the same. Thus, the main difference between the first and the other two possibilities is concerned with the nature of the Sa-factor in 2a-30(Sa), *i.e.* whether it is endogenotic or exogenotic.

Recovery of the transmissibility of the Sa-resistance factor in 2a-30(Sa) by introduction of the R(Sm^{125})-factor from 2a-50(Sm^{125}, Sa) strongly suggests the exogenotic nature of the Sa-resistance factor. However, this does not exclude the following objection: the R-factor carrying the Sm-resistance from 2a-50(Sm^{125}, Sa) may be a peculiar one and pick up the chromosomal gene of Sa-resistance after introduction into 2a-30(Sa). If this were true, the R-factor (Sm^{125}) should pick up other chromosomal markers to the same extent, and hence the frequency of co-transfer of Sm- and Sa-resistance should be much smaller than the values obtained. Though the incidence of co-transfer of Sm- and Sa-resistance was negligible when conjugants were selected on Sm containing medium, all conjugants selected on Sa-medium were doubly resistant, and the number of conjugant colonies on Sa-medium was not so small as on Sm-medium, as can be seen in Table 2. In addition, the number on Sa-medium was even larger than that on Sm-medium when the samples were spread on agar after 2 hours incubation. The remaining possible objection against the concept of the exogenotic nature of the Sa-resistance factor in 2a-30(Sa) is as follows: the R-factor(Sm^{125}) from 2a-50(Sm^{125}, Sa) could only pick up the Sa-resistance gene from the chromosome, because the vacant seat was reserved exclusively for it. The authors can offer no answer to this objection.

Other results more strongly suggesting the exogenotic nature of the Sa-resistance factor of 2a-30(Sa) were the spontaneous loss of Sa-resistance either together with Sm-resistance or independently of it, after introduction of the R-factor (Sm^{125}). A similar phenomenon was also observed in 3a-18gal^-(Sm^{125}, Sa), in which Sa-resistance was lost in a few per cent of the population. If Sa-resistance were expressed by a chromosomal gene, it would be much more difficult to understand the independent or mutual loss with the R-factor (Sm^{125}).

The last experiment described above supports the concept of the exogenotic nature of the Sa-resistance factor in 2a-30(Sa). The Sa-factor, once reactivated and transferred by the R-factor (Sm^{125}) to 3a-18gal^+ and dissociated spontaneously or by acridine treatment from the R-factor (Sm^{125}), was still actively transferred. This paper gives the first description of a transferable R-factor carrying only

Sa-resistance, though the R-factor (Sa) could not be eliminated by acridine treatment. The susceptibility to the acridine treatment is not an indispensable characteristic of exogenotic factors, because prophage and colicigenic factors, which are also regarded to be exogenotic, can not be eliminated by this treatment.

The R-factor (Sa) in 2a-30(Sm^{1000}, Cm, Tc, Sa) and 2a-30(Sa) differs from that of 3a-18gal^+(Sa) in its ability to be transmitted. The former can be regarded as defective in some of elements concerning with the mechanism of transmission and the defect was complemented by coupling with corresponding elements of the R-factor (Sm^{125}) from 2a-50 (Sm^{125}, Sa). When the Sm-resistance was lost from 3a-18gal^+(Sm^{125}, Sa) spontaneously or by acridine treatment, the coupling might be dissociated not by a reversal of the coupling process but in a somewhat different fashion leaving the elements concerning the mechanism of the transmission to the hand of the R-factor (Sa) to complement the defect of the latter. The reactivation of the defective R(Sa)-factor of 2a-30(Sa) by R(Sm^{125})-factor and the acridine induced dissociation of R(Sa)-factor from R(Sm^{125})-factor seem to support the second possibility. However, the third possibility can not be completely excluded.

The loss of the R(Sm^{125})-factor and the R(Sa)-factor shown in Tables 4, 5, 6 is a very interesting phenomenon. However, it can not be explained. The authors can only say that the loss was dependent on the intrinsic characters of the bacteria carrying the R(Sm^{125}, Sa)-factor. The instability and loss of the R(Sm^{125})-factor introduced into 3a-18gal^+, when Sm-resistant conjugants were selected by Sm, was shown to be a cause of the peculiar phenomenon, in which Sm-resistant colonies were most numerous in samples spread just after mixing the two parent cultures. The rapidity of expression of Sm-resistance after introduction into 3a-18gal^+ can be regarded as one of the characteristic markers of the R(Sm^{125})-factor from 2a-50(Sm^{125}, Sa).

The peculiar phenomena described in this paper were all due to the introduction of the R(Sm^{125})-factor from 2a-50(Sm^{125}, Sa). It differed from the R(Sm^{1000}, Cm, Tc, Sa)-factor of 2a-30(Sm^{1000}, Cm, Tc, Sa) not only in the degree and rapidity of phenotypic expression of Sm-resistance and in other drug resistance markers but also in causing various interesting phenomena. The authers can present no explanation for the peculiar character of the R(Sm^{125})-factor and further studies are now in progress.

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